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Physiological Parameters Affecting the Chemosensory Response of *Tetrahymena*

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Abstract. We have investigated the significance of a number of physiological parameters in the preparation of cells for experiments on chemokinesis in *Tetrahymena*. The study comprises (1) growth state of the cells, (2) composition of the starvation medium, (3) concentration of cells during starvation, (4) oxygen saturation of the starvation medium, (5) temperature during starvation, and (6) starvation period. By controlling the physiological state of the cells, we significantly improved the reproducibility of the results obtained in assays for chemokinesis in *Tetrahymena*. In short, cells optimal for chemokinesis at an assay temperature of 28°C should be starved from the exponential growth phase in a concentration below 2×10^5 cells ml^{-1} for 10–20 h. The surface-to-volume ratio of the starvation medium—water or Hepes buffer—should be about 5 cm^{-1} (or more) to ensure more than 95% oxygen saturation of the starvation medium. Maximal chemosensory responses were obtained if the cells were starved at 21°C. The chemokinetic potential of the cells decreased significantly, as did the levels of the ratio of ATP to ADP, if cells were starved at higher temperatures. A tentative correlation between the ATP level in the cells and the chemosensory potential of the cells has been found. We suggest that chemokinesis is a constant quality of *Tetrahymena*, because we found no sign that prolonged starvation or other changes applied to the cells produced an up-regulation of the chemosensory response. Apparently, starvation is obligatory only to remove the growth medium (which is itself a very potent attractant), thereby making the cells sensitive to the chemoattractants.

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Introduction

Chemosensory responses to chemical attractants and repellants are a phenomenon of importance in most cells. Among the ciliate protozoa, chemokinesis has been much studied in genera such as *Paramecium*, *Blepharisma*, and *Tetrahymena* (Van Houten *et al.*, 1981; Leick and Hellung-Larsen, 1992). *Tetrahymena* exhibits positive chemokinesis towards certain peptide hormones and cytokines that also induce positive chemokinesis in specific animal cell types such as leucocytes, muscle cells, and dermal fibroblasts (see review by Leick and Hellung-Larsen, 1992). Furthermore, *Tetrahymena* is a useful model cell for electrophysiological investigations of chemoreception (Ueda and Kobatake, 1977, Tanabe *et al.*, 1980).

Various bioassays for chemokinesis have been applied to *Tetrahymena*, but the results have not been consistent, and day-to-day variations have been reported in many studies (Almagor *et al.*, 1981; Levandowsky *et al.*, 1984; Hellung-Larsen *et al.*, 1986; Leick *et al.*, 1990). We understood that the chemosensory sensitivity of the cells must be dependent on their physiological conditions, but the relationship was obscure.

Tetrahymena is readily obtainable in axenic cultures, and the physiological parameters characterizing populations of dividing and nondividing *Tetrahymena* have recently been described (Hellung-Larsen *et al.*, 1993). We therefore used such cells in a systematic examination of starvation and assay conditions. We have elucidated the relative importance of the physiological parameters involved in the preparation of cells for experiments on chemokinesis in *Tetrahymena*. Furthermore, we have tested a number of physiological conditions not previously controlled in this kind of experiment. Our conclusions about the chemosensory behavior of *Tetrahymena* may be applicable to other organisms.

Materials and Methods

Cell cultures

Tetrahymena thermophila strain B7 was grown axenically in PY medium: 0.75% proteose peptone (Difco), 0.75% yeast extract (Difco), 1.5% glucose, 1 m*M* $MgSO_4$, 50 μM $CaCl_2$, and 100 μM ferric citrate. The PY medium was diluted to ⅓ in water prior to use. Fifty-milliliter cultures of B7 cells were grown at 35°C in 500-ml Fernbach flasks. The cultures were kept unshaken to avoid cell division stress (Hellung-Larsen and Lyhne, 1992). Cells in exponential growth phase were used to inoculate the cultures to a concentration of about 10^4 cells ml^{-1}. Transfer to starvation medium was performed after 16–20 h at a cell density of about 8×10^5 cells ml^{-1} (late exponential growth phase) or 40–50 h at a cell density of approximately 1.3×10^6 cells ml^{-1} (early stationary phase). For starvation, the cells were collected by centrifugation at $500 \times g$ for 3 min and then gently resuspended in the applied starvation medium (10 m*M* Hepes or deionized water). After starvation, the cells were used in the assay without further handling (resupension, dilution, etc.). All materials and media involved in the growth and starvation of the cells were sterilized by pressure sterilization (2 atm, 120°C, 30 min). Cell concentration and cell volumes were estimated with the aid of a Coulter Multisizer, as previously described (Hellung-Larsen and Andersen, 1989).

Oxygen tension

O_2 tension was measured by use of a microelectrode (WTW, Weilheim, Germany) or an acid-base analyzer (Radiometer ABL 30, Copenhagen) and expressed in percentage of maximum saturation in distilled water at 21°C.

ATP and ADP determinations

The ribonucleotides were analyzed by the high-performance liquid chromatography (HPLC) technique. A neutralized cellular PCA extract (100–200 μl) was applied to a strong anion exchange column (Partisil-10 SAX, 2 mm $\phi \times 200$ mm, Whatmann, Clifton, NJ). The column was eluted for 11 min with 0.005 *M* KH_2PO_4, pH 5.5, followed by a gradient for 14 min to 0.25 *M* KH_2PO_4 pH 4.5; an isocratic period was allowed for 10 min, and the column was re-equilibrated with the starting buffer before a new sample was added. The absorbancy at 254 nm was recorded, and the peaks were automatically integrated. Further details of the procedure are described elsewhere (Klenow and Ostergård, 1988). All PCA extractions were performed in duplicate and the standard error of the obtained pool sizes of ribonucleotides was less than 5%.

Swimming speed

Swimming speeds were determined in the laboratory of Professor Donat-P. Häder, Friedrich-Alexander Universität, Erlangen, Germany. About 2 ml of cell suspension was gently pumped into a circular vertical observation chamber positioned on a microscope, and the cell movement was recorded by a CCD video camera (LDH 600, Philips, Hamburg). A computerized analysis of swimming speeds was performed (Häder and Lebert, 1985). Each estimation was based on 2000 tracks or more, and the standard deviations were less than 10%.

Capillary assay

A capillary technique with single glass capillaries was used. Heparinized glass capillaries (75 mm long, inner diameter 1.1–1.2 mm) were filled with test solution, and one end of the capillary was sealed with wax. The capillary was placed horizontally through a sealed hole into the jar containing the cell suspension, and the open end of the capillary was brought in contact with the cell suspension. The assays were incubated for 45 min at 28°C. All experiments were carried out in quintuplicate. After incubation, the number of cells accumulated in the capillaries was determined by microscopic counting. Each capillary was emptied into a small test tube and 2 μl of an 0.2% aqueous solution of crystal violet was added to each sample. Subsequently, cells from 2×1.8 μl of each sample were counted in a hemacytometer. The cell concentrations in the capillaries are expressed as a percentage of the cell concentration (of the cell suspension) used in the particular assay.

Experimental setup

The basic experimental design is shown schematically in Figure 1. Exponentially growing cells or cells grown to the early stationary phase were transferred to starvation medium at a concentration of exactly 5×10^4 cells ml^{-1}. The cell suspension was divided into aliquots as follows: 50 ml in each of four 500-ml Fernbach flasks (a); 80 ml in each of four 250-ml Ehrlenmeyer flasks (b); 80 ml in each of four 100-ml Ehrlenmeyer flasks (c); and finally, four 100-ml Ehrlenmeyer flasks filled to the top with the cell suspension (d).

The surface-to-volume ratios (S/V) were calculated to be 4.8 cm^{-1} (a), 0.6 cm^{-1} (b), 0.2 cm^{-1} (c), and 0.03 cm^{-1} (d). As indicated in Figure 1, one of each "starvation system" was incubated for starvation at 15°, 21°, 28°, and 35°C. The chemosensory response and a number of physiological parameters were determined after 16 h of starvation (16 h was chosen because initial experiments had revealed this period of starvation to be optimal; nevertheless, we reinvestigated the importance of starvation and starvation period once the other variables were investigated, as described in the Results section titled *Starvation period*). The whole experiment was performed four times; a representative experiment is shown in Table I.

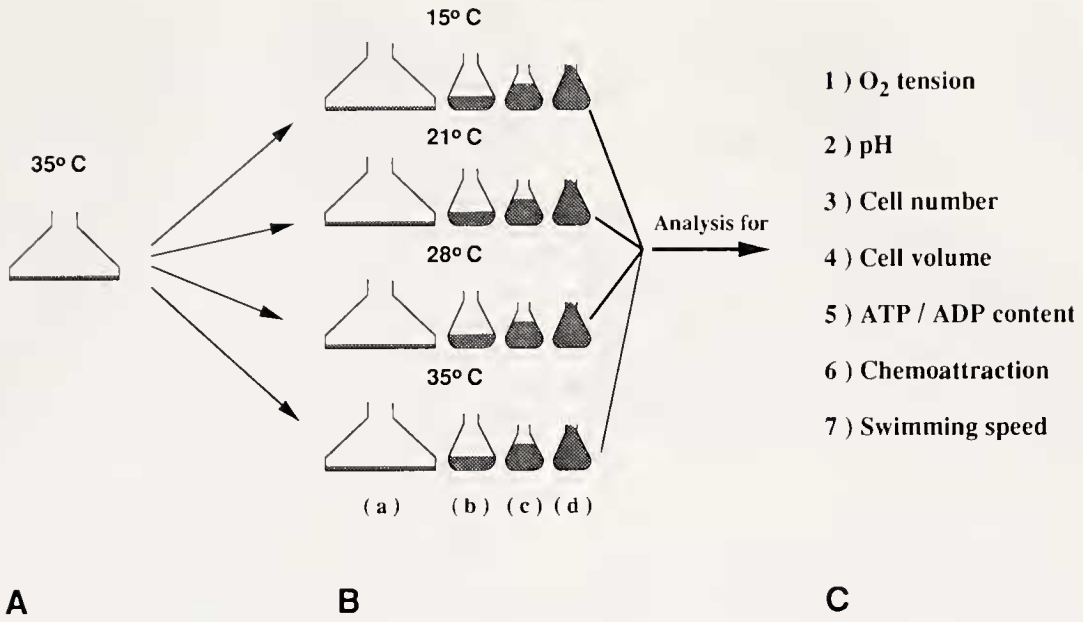

Figure 1. The experimental setup giving rise to different oxygen tensions (a, b, c, d). Cells grown at 35°C in Fernbach flasks (A) were transferred to starvation at a cell concentration of 5×10^4 cells ml^{-1}. Initially, Hepes-KOH buffer, pH = 7.4, was used as the starvation medium. The cell suspension was divided into the following aliquots: 50 ml in each of four 500-ml Fernbach flasks (a), 80 ml in each of four 250-ml Ehrlenmeyer flasks (b), 80 ml in each of four 100-ml Ehrlenmeyer flasks (c), and four 100-ml Ehrlenmeyer flasks filled to the top with the cell suspension (d). As indicated in (B), the cells were incubated in the starvation medium at 15°, 21°, 28°, and 35°C, respectively. The chemoattraction and a number of physiological parameters were determined after 16 h of starvation (C). The results of a representative experiment are summarized in Table I.

For the quantitative analysis of the chemosensory response of the cells, we used a capillary assay as illustrated in Figure 2. Proteose peptone (PP) in a concentration of 1 mg ml^{-1} was used as the attractant. All cell suspensions were maintained at 28°C for half an hour before the start of the assay. The assays were incubated for 45 min at 28°C. The assay temperature was chosen in accordance with earlier findings (Leick *et al.*, 1990). Recent studies have confirmed that the optimal temperature for chemokinesis in *Tetrahymena thermophila* is about 28°C (Koppelhus *et al.*, 1994). Five assays testing the effect of proteose peptone and five control assays were performed simultaneously. The mean values are listed in Table I and the standard deviations were calculated and listed for some selected measurements.

Results

We investigated the influence of the following physiological parameters in the preparation of *Tetrahymena* cells for experiments on chemosensory behavior: (1) growth state of the cells, (2) composition of the starvation medium, (3) cell number during starvation, (4) oxygen saturation of the starvation medium, (5) temperature during starvation, and (6) starvation period.

Influence of growth state of the cells

In the experiment summarized in Table I, cells were starved from exponential growth phase. Essentially identical results were obtained whether cells were starved from the late exponential growth phase or the stationary phase. As indicated in Table I, the cell concentration increased during starvation. We found that cells starved from logarithmic growth phase give rise to an increase in cell concentration of 10–30%, whereas cells starved from late logarithmic growth phase or stationary growth phase give rise to only a small, if any, increase in cell concentration. These findings indicate that cells at some stage of division will complete the initiated division even after transfer to the starvation medium.

Influence of starvation medium

We assumed that the pH of the medium would be affected if the starvation medium had no buffer capacity. Initially, we used 10 m*M* Hepes-KOH, pH 7.4 (Hepes) for the starvation medium because we recently found that long-term starved cells survive better in Hepes than in 10 m*M* Tris-HCl, pH 7.4 (Tris) (Hellung-Larsen *et al.*, 1993). As shown in Table I, pH was 7.4 after starvation

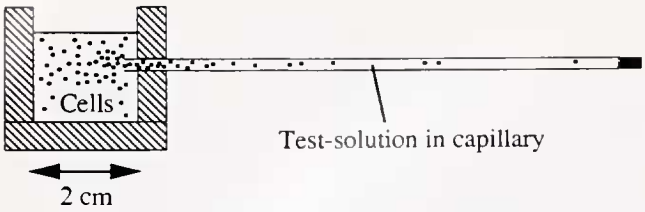

Figure 2. A schematic presentation of the capillary assay. Proteose peptone (PP) in a concentration of 1 mg ml^{-1} was placed in the capillary and one end of the capillary was sealed with wax. The capillary was then placed horizontally through a sealed hole into the jar containing 2.5 ml of cell suspension, and the open end of the capillary was brought in contact with the cell suspension. Incubation was usually for 45 min at 28°C, and the assays were scored by microscopic determination of the cell concentration in the capillary. Parallel assays containing only the solvent were performed as controls.

in Hepes. The minor decrease in pH observed in the most hypoxic cell suspensions was probably due to accumulation of lactic acid. When Hepes is used as starvation medium, it must also be used as diluent in the assays because we found that cells starved in Hepes swam more slowly than cells starved in deionized water (results not shown). This effect is probably due to the presence of K^+ in the buffer. K^+, as well as other cations, is known to influence the swimming speed of ciliate protozoa (Nakaoka *et al.*, 1983). To minimize kinetic effects of cations in the starvation medium, we attempted to starve cells in deionized water. Surprisingly, water proved to be an excellent starvation medium, and the chemosensory sensitivity of the cells was even higher than that observed using Hepes or Tris for starvation. Furthermore, the pH in cell suspensions starved in deionized water did not decrease if the O_2 tension was kept above 60% (results not shown).

Influence of the cell concentration during starvation

To illuminate the significance of the cell concentration during starvation, we starved cells at concentrations of 2.5×10^4, 5.0×10^4, 10×10^4, 20×10^4, and 40×10^4 cells ml^{-1}. The cells were starved at 21°C and S/V's were 4.8 cm^{-1}.

Cell suspensions of 2.5×10^4, 5.0×10^4, 10×10^4, and 20×10^4 cells ml^{-1} revealed 100% saturation of O_2 after starvation, whereas the saturation of O_2 decreased to 91% in the cell suspension of 40×10^4 cells ml^{-1}. In the cell suspensions having 100% saturation of O_2, we found that the chemosensory sensitivity toward PP was unaffected by the concentration of cells during starvation, as the responses were almost identical (SD less than 5%). Studies in another assay—the improved two-phase assay—revealed that sensitivity towards weak attractants such as certain amino acids was optimal if cells were starved at a concentration not exceeding 2×10^5 cells ml^{-1} (Koppelhus *et al.*, 1994).

Influence of temperature during starvation

As indicated in Table 1, the responses toward PP were significantly higher when cells were starved at 15° or 21°C than when cells were starved at 28° or 35°C. In contrast, the background level in the assay was highest when cells were starved at the highest temperatures. The ATP/ADP ratio decreased when the temperature was increased during starvation. The ATP pools of cells starved for 16 h were 1–4 nmol 10^{-6} cells. In comparison, the ATP pools of exponentially growing cells were about 20 nmol 10^{-6} cells (results not shown). The temperature of starvation also influenced the volume of the cells: the cells became smaller as a function of increasing temperature during starvation. At the smallest S/V, the cell volume decreased even more. Because of the indirect electronic volume determination applied, all cell suspensions were examined microscopically to confirm the qualitative conclusions. Microscopic examination revealed that the anoxic cells were somewhat flattened, and the divergence in cell volume compared to cells starved at the same temperature might be due to the osmotic state of the cells.

Influence of oxygen saturation of the starvation medium

The results in Table 1 reveal the precise dependence of oxygen saturation on the S/V of the starvation medium. The saturation of oxygen was essentially 100% if cells were starved under conditions in which S/V was equal to 4.8 cm^{-1}.

Suspensions having S/V equal to 0.6 cm^{-1} became more or less hypoxic during starvation, whereas the cells in suspensions having S/V equal to 0.03 cm^{-1} became strongly hypoxic (anoxic) during the period of starvation, which in turn killed the cells. The survivors in these cell suspensions had highly irregular swimming patterns. Disregarding the strongly hypoxic cell suspensions (*i.e.*, the (d) suspensions), we found that the lower the O_2 tension of the cell suspension, the greater the responses toward PP. The background level also increased in relation to lower O_2 tension of the cell suspension, but in general the background level did not increase in direct proportion to the response toward PP; as a result, signal-to-noise ratios were augmented (Table 1).

We found no significant differences in swimming speed of cells between cultures starved into self-induced hypoxia and suspensions that maintained full oxygen saturation during starvation. The swimming speeds were between 0.4 and 0.5 mm s^{-1} regardless of the oxygen saturation of the starvation medium. This also seems to indicate that there is no direct relationship between the ATP pools of the cells and their ability to maintain their swimming speed. For comparison, the swimming speeds of exponentially growing cells in culture are between 0.5 and 0.6 mm s^{-1} (Hellung-Larsen *et al.*, 1993).

Table I

Different physiological parameters, including chemosensory response, of Tetrahymena *starved under different conditions*

Temperature during starvation (°C)	Surface/volume ratio of starvation medium (cm^{-1})	O_2 tension of medium after starvation (%)	pH in medium after starvation (%)	Cell conc. after starvation (cells ml^{-1})	Cell volume after starvation (μm^3)	ATP/ADP (nmol 10^{-6} cells)	Capillary containing 1 mg PP ml^{-1} (signal) (% of original cell concentration)	Capillary containing no attractant (noise) (% of original cell concentration)	Signal/noise ratio
15	4.80 (a)	100	7.4	55,000	3500	3.2/0.8	96 ± 4	11 ± 2	9
	0.60 (b)	99	7.4	52,000	3500	ND	98 ± 4	8 ± 3	12
	0.20 (c)	42	7.4	55,000	3500	ND	143 ± 12	8 ± 3	18
	0.03 (d)	9	7.2	47,000	3300	ND	41 ± 8	5 ± 1	8
21	4.80 (a)	100	7.4	62,000	3000	2.4/0.5	99 ± 6	11 ± 2	9
	0.60 (b)	96	7.4	62,000	3000	2.3/0.4	128 ± 6	11 ± 2	12
	0.20 (c)	42	7.4	61,000	3000	2.3/0.6	165 ± 10	14 ± 1	12
	0.03 (d)	8	7.2	28,000	2500	0.4/2.3	62 ± 9	2 ± 1	31
28	4.80 (a)	100	7.4	64,000	2500	1.6/0.3	51 ± 5	12 ± 2	4
	0.60 (b)	95	7.4	62,000	2500	ND	74 ± 6	12 ± 3	6
	0.20 (c)	40	7.3	63,000	2500	ND	95 ± 7	14 ± 1	7
	0.03 (d)	8	7.1	20,000	2000	ND	15 ± 2	0 ± 0	—
35	4.80 (a)	98	7.4	63,000	1800	1.2/0.4	42 ± 4	15 ± 1	3
	0.60 (b)	84	7.4	63,000	1800	ND	63 ± 4	17 ± 5	4
	0.20 (c)	35	7.2	63,000	1800	ND	79 ± 6	17 ± 2	5
	0.03 (d)	8	7.0	13,000	1000	ND	5 ± 1	0 ± 0	—

Exponentially growing cells were starved at a concentration of 5×10^4 cells ml^{-1}. The cells were starved at different temperatures and at different S/V ratios (*cf.* Fig. 1). After starvation for 16 h, the O_2 tension, pH, cell concentration, cell volume, ATP/ADP, and chemosensory response were measured. The assays on chemokinesis were performed in quintuplicate, and mean values ±SD are given. ND = not determined.

Starvation period

Previous studies have suggested that starvation of *Tetrahymena* cells was necessary to obtain a chemosensory response (Levandowsky *et al.*, 1984). To investigate the chemoattraction of the nonstarved cells, we used deionized water to dilute a suspension of exponentially growing cells from a concentration of 6×10^5 cells ml^{-1} to a concentration of 5×10^4 cells ml^{-1}. Subsequently, we tested these nonstarved cells in the capillary assay and found a response toward PP of 85% and a background level of only 1%. These lower values of accumulation in comparison with cells starved for 16 h may be due to a competitive chemoattractive effect from the leftover growth medium within the cell suspension—the growth medium is itself a potent chemoattractant because of its PP content. This was further confirmed (results not shown) by the fact that addition of growth medium (or PP) to suspensions of starved cells suppressed the accumulation of cells in the assay according to the concentration of the added medium (or PP).

In another experiment, cells were starved at 5×10^4 cells ml^{-1} at 21°C in deionized water with an S/V of 4.8 cm^{-1}, and aliquots of the cell suspension were tested in the capillary assay after starvation for 1 day (16 h), 3 days, 5 days, and 7 days. The responses toward PP (1 mg/ml) were found to be 100 ± 7%, 92 ± 4%, 71 ± 5%, and 59 ± 2%, respectively. The accumulations in the respective control assays were 11 ± 2%, 9 ± 2%, 5 ± 1%, and 4 ± 1%. That is, the accumulation in the assays (both with and without PP) gradually decreased as the starvation of the cells proceeded. This effect may be a simple consequence of the gradual decrease in swimming speed known to take place during starvation (Hellung-Larsen *et al.*, 1993).

Discussion

The main purpose of this study was to determine the optimal physiological conditions for chemoattraction studies with *Tetrahymena*. The data obtained suggest that the cells should be starved for about 16 h at about 21°C, and that the surface-to-volume ratio of the cell suspension should be no less than 4.8 cm^{-1}. Furthermore, the cells should be starved at a concentration below 2×10^5 cells ml^{-1}. Deionized water proved to be a suitable starvation medium. Subsequent studies have revealed that cells starved in this way are indeed suitable for the detection of even weak attractants such as certain amino acids and peptides (Koppelhus *et al.*, 1994).

We did not find the increase in swimming speed observed by Nelsen and Debault (1978) in response to starvation. Some explanation for this discrepancy may be that Nelsen and Debault used "Dryls medium" containing 2 m*M* of $CaCl_2$ for the starvation medium. $CaCl_2$ at this concentration is known to hyperpolarize the cells, making them swim faster and more smoothly (Machemer, 1989).

Jauker and coworkers found a rate of decrease in ATP pools in *Tetrahymena* of 1–3 nmol h^{-1} after cells were transferred to starvation at room temperature (Jauker *et al.*, 1986). Our results seem to confirm these findings. Furthermore, the ATP decrease during starvation seems to be closely correlated to the applied temperature. When using cells starved at 21°, 28°, and 35°C, we found an interesting correlation between the ATP pools of the cells and their chemosensory responses. Higher levels of ATP gave better chemosensory responses than did low levels. The correlation is not straightforward, however, because cells starved at 15°C had similar chemosensory responses to cells starved at 21°C, even though they have larger pools of ATP.

We suggest that the nonoptimal responses obtained using nonstarved cells are due to a competitive effect of chemoattractants within the cell suspension. It might be preferable if cells were deprived of growth medium without any period of starvation (and consecutive loss of ATP). This might be done by applying an electric field to a cell culture and separating the cells from the medium by means of galvanotaxis.

We found no obvious sign of an up-regulation of the chemosensory response after prolonged starvation. This seems to indicate that the ability for chemosensory behavior in *Tetrahymena* is a constant quality of the cells and that starvation is obligatory only to remove the growth medium, which is a very potent chemoattractant. Furthermore, a certain period of starvation seems necessary for the cells to overcome the stress (ciliary damage, etc.) of being centrifuged. Without the possibility of completely separating growing cells from their medium in a manner that does not affect the cells, it seems impossible to further investigate the importance of short-term starvation on the chemosensory response of the cells.

The augmented responses observed when hypoxic cell suspensions were assayed suggest that *Tetrahymena* may exhibit a positive aerotactic behavior. Aerotactic responses are observed in a number of protozoa and bacteria and may be of vital importance in the survival of motile microorganisms (see review by Levandowsky and Hauser, 1978).

The results presented in Table I demonstrate that signal-to-noise ratios can be misleading as a quantitative measurement of the chemosensory response; thus, they must be considered inadequate if the cell material is not strictly standardized and controlled. Identical cells should be used in assays for the comparison of attractants.

Considering the capillary assay used for this study, we found that even with optimal cell material, the assay should be performed in at least quintuplicate to ensure statistical significance. This makes it difficult to test

more than a few samples at a time. For the purpose of testing a large number of samples, we have developed a sensitive and easy-to-perform spectrophotometric assay, which is described in the accompanying paper (Koppelhus *et al.*, 1994).

Acknowledgments

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